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(54) Title: PROTEINACEOUS ANTI-DENTAL PLAQUE AGENTS

(57) Abstract

The development of dental plaque is inhibited by blocking bacteria/pellicle binding sites or by inhibiting the action of plaque-forming enzymes, in either case by means of a novel binding protein. A population of muteins is generated by extensive random mutagenesis of selected codons of a gene encoding a suitably stable protein, e.g., aprotonin. The muteins are expressed by a phage, bacterial cell or bacterial spore and displayed on its outer surface. Displayed muteins which bind to a pellicular protein, such as a proline-rich protein or statherin, or to a plaque-forming bacterial enzyme, such as a streptococcal glucosyltransferase, are isolated by affinity chromatography. The pellicle binding proteins may be used directly, or to tether a GTF inhibitor protein.

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PROTEINACEOUS ANTI-DENTAL PLAQUE AGENTS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the genetic engineering of novel proteins useful as anti-dental plaque agents.

Information Disclosure Statement

The hydroxyapatite (HAP) mineral comprising the tooth surface is coated with a membrane of protective protein called the pellicle (Fig. 1a). The pellicle is composed of simple proteins (those containing only the normal amino acids), glycosylated proteins (those also containing short polymers of sugars called oligosaccharides which are covalently attached to the amino acids of the proteins) and phosphorylated proteins (Reddy, et al., 1985).

Dental plaque is a collection of bacteria of many types which can number in the billions and are attached to the teeth through the pellicle. Two types of pellicle proteins are of special importance, the proline-rich proteins (PRPs) and statherin, because bacteria initially bind to the pellicle using receptor sites on these proteins. Bacteria also bind to oligosaccharide receptors on the pellicle glycoproteins.

The plaque formation process is shown in simple terms in Figure 1. In Fig. 1a, the PRP or statherin receptors are illustrated schematically as circles and ellipses and the oligosaccharide receptors are illustrated as pentagons and hexagons. In Fig. 1b, two different types of bacteria bound to a PRP receptor and a polysaccharide receptor are illustrated. The bacteria bind to the receptors using proteins on their surface called adhesins which "recognize" the receptors by a shape-dependent mechanism (often called a "key-and-lock" mechanism) as illustrated schematically in the figure.

The next steps in plaque formation involve several processes: the increase in numbers of bacteria by cell doubling, coaggregation (sticking together) of similar and different types of bacteria, and the spinning of a three-dimensional polysaccharide matrix (a large web-like polymer of sugars) by some of the bacteria. The polysaccharide matrix acts like a three-dimensional web to which bacteria can attach and through which bacteria cannot pass because they are too big (Kolenbrander, 1988; Gibbons and van Houte, 1980).

Both coaggregation and matrix formation are mechanisms for the bacteria to remain attached to the pellicle even in the presence of saliva flow and other mechanical forces in the mouth which tend to wash out the bacteria. The result of receptor binding, coaggregation and web formation is plaque, which is illustrated in Fig. 1c.

Dating back to the 1960s, there have been numerous clinical studies on the efficacy of mouthwashes and dentifrices containing dextranase enzymes to destroy (hydrolyze) the polysaccharide matrix. Despite the large number of studies, the results were inconclusive regarding the ability of these enzymes to reduce plaque or prevent cavities. (Some typical studies and analyses of dextranase-containing mouthwashes are: Lobene, 1971; Caldwell, et al., 1971; Keyes, et al., 1971; Koga, et al., 1986; Hull, 1980; Lobene, 1979). For patents on dextranase or other carbohydrase-containing compositions, see Pader, U.S. 4,082,841, Stoudt, U.S. 4,335,101, Simonson, U.S. 4,328,313, Ioka, U.S. 4,469,673, Miller, U.S. 3,630,924, Shimada, U.S. 4,438,093, and Woodruff, U.S. 3,686,393. It is probably safe to conclude that the efficacy of these enzymes used under the conditions of the studies was not great enough to reduce plaque sufficiently to provide a benefit a consumer could perceive. Later, we will discuss some factors which may be important for efficacy and which could not be accounted for in these studies, but can now be addressed using the present invention.

Dentifrices containing glucose oxidase and amyloglucosidase may have some efficacy, at least for reduction of gingivitis, according to one recent Study (Midda and Cooksey, 1986). These enzymes produce hydrogen peroxide (H202) from fermentable carbohydrates metabolized by the bacteria. In saliva, this H_2O_2 combines with thiocyanate (HSCN) to produce potent low molecular weight chemicals toxic to bacteria. This reaction is catalyzed by salivary lactoperoxidase. The chemical equation is:

lactoperoxidase

H202 + HSCN ----------> OSCN⁻

As will be discussed later, one factor which may account for apparent efficacy of glucose oxidase and amyloglucosidase dentifrices is that the toxins are low molecular weight compounds and thus are able to diffuse through the plaque to the bacteria, their site of action. Pertinent patents include Hoogendoorn, U.S. 4,178,362 and Pellico, U.S. 4,578,265.

Other enzymes have also been used for this purpose. For use of proteases to attack the plaque, see Weeks, U.S. 3,696,191, Pader, U.S. 4,082,041, Nachtigal, U.S. 3,701,830 and Colonder, U.S. 4,058,595. For invertases, see Ishibashi, U.S. 4,107,291 and Becker 3,733,399. For lipases, see Pader, U.S. 3,855,142 and U.S. 4,082,041.

Many other compounds have been tested in toothpastes and mouthwashes for ability to reduce plaque. A very recent study (Moran, et al., 1989) compared several commercial toothpastes containing different antimicrobials, hexetidine/zinc citrate, triclosan, amyloglucosidase/glucose- oxidase. These toothpastes were compared to a simple fluoride-containing toothpaste. In the study, no differences in either plaque index or gingivitis between the antimicrobial toothpastes and the fluoride toothpaste were found. Again, factors which the technology of the present invention can address were not accounted for.

One antimicrobial compound, chlorohexidine, is an effective antiplaque agent (Moran, et. al., 1988; Johnson and Rozanis, 1979). Its effectiveness is thought to derive in part from the fact that it adheres to the teeth and therefore has greater residence time in the mouth than the others. However, chlorohexidine discolors teeth, a negative product feature.

There are several factors not accounted for in the dextranase and amylase studies, one or more of which could have affected the efficacy of these enzymes and indeed any protein or non-protein anti-plaque agent. In addition, analysis of these factors may explain the apparent greater efficacy of oral preparations containing glucose oxidase and amyloglucosidase. These factors, all of which are potential problems to be overcome via appropriate protein engineering, are summarized below:

1. Washout: Proteins, especially enzymes, must remain in the mouth to be effective. Enzymes are not consumed in the chemical reactions they catalyze, so derive their effectiveness from being used over and over to catalyze the same reaction. When included in a mouthwash or dentifrice, they are usually washed out of the mouth within a minute or so after introduction, so little of their potential benefit is realized.

2. Penetration of plaque: Plaque is a dense three dimensional matrix and is not penetrated by most proteins. Thus, enzymes such as dextranases can only hydrolyze the available surface components of the solid-phase matrix. To destroy effectively the whole matrix, it must be hydrolyzed internally as well as on the surface.

3. Host immune response: Proteins which are not native in humans can evoke antibody production which could reduce efficacy or cause allergic reactions. At present, the enzymes used in dentifrices and mouthwashes are those commercially available and are not natural human proteins. An example of where this has turned out to be a problem is enzyme detergents where some people have developed skin

rashes. Allergies may be less of a problem with ingested proteins because of the wide range of proteins ingested from food.

4. Incompatible oral environment: The enzymes used in dentifrices and mouthwashes were picked from those commercially available. Their optima for catalytic activity with respect to pH, temperature, and salt concentration are expected to be different from the conditions present in the mouth. Also, proteases produced by oral bacteria can degrade proteins.

5. Product shelf life: The time period from manufacture to sale could be long. Products may be shipped, stored and displayed at widely varying temperatures. Some proteins may become inactive in aqueous solutions after a long time or at extreme temperatures.

The efficacy of dextranase preparations could have been influenced by any or all of these five factors. The products of glucose oxidase and amyloglucosidase, on the other hand, would not be affected by the second factor because the toxic molecules produced by these enzymes have low molecular weight and can diffuse rapidly into the plaque. (It is interesting to note that enzymes produced by wood-rot fungi to degrade the lignin in wood also work by producing low molecular weight radicals which diffuse into the wood to their lignin targets. Wood is even more dense than plaque, so it would be difficult for the large ligninase enzymes to contact their targets directly.) In addition, the toxin produced is potent. Hypothiocyanite (OSCN-) interferes with the oxidation reduction glycolytic enzymes which contain essential thiol groups.

Simonson, U.S. 4,138,476 stated that while dextranases will bind to hydroxyapatite at lower acidic pH, the retention of these enzymes may be diminished in the mouth by the coating of the teeth with proteins, mucins, or other organic films from saliva, dental plaque, etc., and by the tendency for the pH of the oral fluid to rise to the 6.2 to 7.4 range. As a result of these factors, the dextranases

adhere loosely and are cleared from the tooth surfaces by salivary flow and dietary liquids in a matter of minutes. Simonson suggested crosslinking the dextranase to a carrier, e.g., phosphoserine or phosvitin, having a higher affinity for the hydroxyapatite components of the tooth surface than does the dextranase. Such a complex would not be able to bind to the proteins of the pellicle and would not displace bacteria from the pellicular binding sites.

Hay et al. (US patent 5,013,542, issued May 7, 1991) discloses a method of inhibiting the adhesion of micro-organisms to teeth; the method involves replacing the normal PRPs which are part of pellicle with fragments of PRPs which bind hydroxyapatite but to which oral bacteria can not bind. Our most preferred method, however, differs from their method in that we allow formation of normal dental pellicle and then cover the sites recognized by oral bacteria with a novel, high-affinity binding protein.

SUMMARY OF THE INVENTION

The present invention is intended to overcome the deficiencies of the prior art. More particularly, the present invention contemplates the preparation of novel binding proteins which interfere with the process of plaque formation. These binding proteins fall into the following categories:

1. Binding proteins to block the binding of plaque bacteria to pellicle proteins such as PRPs.

2. Enzyme inhibitor proteins which bind to and block an enzyme from carrying out its catalysis.

3. Tethering proteins to which an effective anti-plaque agent is attached; the tethering proteins prevent washout of the agent from the mouth.

These novel binding proteins are prepared and identified by adaptation of a method for the generation and selection of novel binding proteins described in commonly owned Ser. No. 07/240,160 (WO90/02809), incorporated by reference herein. In essence, a gene encoding a known

protein is semi- extensively mutagenized at codons that specify amino acids the side groups of which are exposed on the surface of the parental protein, to yield millions of mutant genes, each encoding a mutant protein. This gene is placed under appropriate control so that the protein is expressed and transported to the outer surface of a phage, cell or spore ("genetic package") bearing the corresponding gene. The genetic packages are then screened for their ability to bind to the desired target, e.g., a pellicle protein or hydroxyapatite beads coated with one or more pellicle proteins. The successful packages are propagated and studied, and perhaps subjected to further rounds of mutation and screening. Note that an important feature of this approach is that the binding protein remains physically associated with the corresponding gene. Once an acceptable protein is identified, its corresponding gene may be cloned into an expression system suitable for large-scale production.

The present invention addresses each of the factors identified above as reducing the efficacy of proteinaceous anti-plaque agents, as shown in Table I below:

Table I. Factors Influencing the Efficacy of Proteins in Mouthwashes, Toothpastes and Other Dental Compositions.

Factor	How addressed
Washout	Tether proteins to pellicle or plaque. Use time-release tethers or leashes.
Penetration of Plaque	Use small proteins or enzymes or those whose active agents are small molecules.
Host immune response	Use proteins which are close to native in humans.
Incompatible oral environment stability.	Use site-directed mutagenesis to adjust optima and increase
Product shelf life	Use stable "scaffold" proteins. Select stable proteins from many candidates.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the plaque formation process.

Figure 2 illustrates how binding proteins may competitively inhibit the binding of bacteria to PRP receptors.

Figure 3 depicts how enzymes convert a substrate to a product (3a) and how an inhibitor blocks formation of the enzyme-substrate complex (3b).

Figure 4 compares untethered and pellicle-tethered anti-plaque agents.

Figure 5 illustrates two different forms of tethered agents.

Figure 6 discloses the amino acid sequence of a family of proline-rich proteins.

Figure 7 illustrates inhibition of the S. mutans GTF enzyme.

Figure 8 depicts an expression cassette suitable for expression of aprotonin-derived proteins in E. coli.

DETAILED DESCRIPTION OF THE INVENTION

PRP-Binding Protein

In one embodiment, a binding protein is developed which binds more strongly to PRPs (or like targets) than do the bacterial adhesins, to prevent plaque bacteria from binding to the PRPs or to dislodge plaque bacteria from the PRPs.

The proline-rich proteins (PRPs) and statherin have been well characterized with respect to their binding both to teeth and plaque bacteria (Gibbons and Hay, 1988 and 1989). The amino acid sequences for six PRPs have been determined (Hay, Bennick, Schlesinger, Minaguchi, Madapallimattam, Schluckbier, 1988), along with that for the protein statherin which has related activity (Schlesinger and Hay, 1977). Since the PRPs and not statherin are thought to be the major proteins to which plaque bacteria bind, the following discussion will be centered on the PRPs, although most of what is said is applicable to any protein

with similar activity.

In Fig. 6, the amino acid sequence of PRP-1 is presented. In the bottom of the figure is a diagram showing the sequence differences among the six PRPs, denoted PRP-1 through PRP-6. The PRPs are from 106 to 150 amino acids long. They have two phosphoserines at one end which are mainly responsible for binding to HAP (Bennick, et al., 1979; Hay and Moreno, 1979). At the other end of the PRPs, receptors are responsible for selectively binding plaque bacteria. The particular species which binds depends on the amino acid sequence in the receptor region. In the instant example, we would make a binding protein which binds more strongly to these regions than do the adhesins of the plaque bacteria.

As taught in Ser. No. 240,160 (WO90/02809), we begin by extensively mutagenizing ("variegating") a gene encoding a stable, well characterized protein so that many codons specifying surface amino-acid residues are varied. The term "variegated DNA" (vgDNA) refers to a mixture of DNA molecules of the same or similar length which, when aligned, vary at some codons so as to encode at each such codon a plurality of different amino acids, but which encode only a single amino acid at other codon positions. It is further understood that in variegated DNA, the codons which are variable, and the range and frequency of occurrence of the different amino acids which a given variable codon encodes, are determined in advance by the synthesizer of the DNA, even though the synthetic method does not allow one to know, a priori, the sequence of any individual DNA molecule in the mixture. The number of designated variable codons in the variegated DNA is preferably no more than 20 codons, and more preferably no more than 5-10 codons. The mix of amino acids encoded at each variable codon may differ from codon to codon. A population of genetic packages into which variegated DNA has been introduced is likewise said to be "variegated".

From time to time, it may be helpful to speak of the

"parent sequence" of the variegated DNA. When the novel binding domain sought is an analogue of a known binding domain, the parent sequence is the sequence that encodes the known binding domain. The variegated DNA will be identical with this parent sequence at one or more loci, but will diverge from it at chosen loci. When a potential binding domain is designed from first principles, the parent sequence is a sequence which encodes the amino acid sequence that has been predicted to form the desired binding domain, and the variegated DNA is a population of "daughter DNAs" that are related to that parent by a recognizable sequence similarity. By extension, the binding domain or protein encoded by the parental DNA sequence is the "parental" binding domain (PPBD) or protein of the family of potential binding domains or proteins encoded by the variegated DNA.

For the purposes of this invention, the term "potential binding domain" (PBD) refers to a binding domain of a protein encoded by one species of DNA molecule in a population of variegated DNA wherein the region of variation appears in one or more subsequences encoding one or more segments of the polypeptide having the potential of serving as a binding domain for the target substance. The potential binding proteins are "variegants" (a family of mutants having a particular pattern of variation) of the parental binding protein. The abbreviation SBD is used for potential binding domains which succeed in binding the target.

Preferably, the variegation is such as will cause a typical transformant population to display 10^6-10^7 different amino acid sequences by means of preferably not more than 10-fold more (more preferably not more than 3-fold) different DNA sequences.

A "replicable genetic package" is a virus, cell or spore which replicates and expresses the binding domain-encoding gene, and transports the binding domain to its outer surface. The binding domain is usually expressed and transported in the form of a chimeric protein, which may be processed after expression. A "chimeric protein" is a

fusion of a first amino acid sequence (protein) with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of the first protein. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergeneric", etc. fusion of protein structures expressed by different kinds of organisms. One amino acid sequence of the chimeric proteins of the present invention is typically derived from an outer surface protein of a "genetic package", such as a phage, bacterium or spore. The second amino acid sequence is one which, if expressed alone, would have the characteristics of a binding protein (or a binding domain thereof) but is incorporated into the chimeric protein as a recognizable domain thereof. It may appear at the amino or carboxy terminal of the first amino acid sequence (with or without an intervening spacer), or it may interrupt the first amino acid sequence. The first amino acid sequence may correspond exactly to a surface protein of the genetic package, or it may be modified, e.g., to facilitate the display of the binding domain.

In the present invention, The "parental" or "scaffolding" protein used as a "reference" for the mutation-and-selection strategy need not have any PRP-binding activity. Potential "parental" proteins include aprotinin (58AA; 3 -SS-), HI-8e domain of human inter-alpha trypsin inhibitor (58AA; 3 -SS-), crambin (46 AA; 3 -SS-), alpha purothionin (45 AA; 4 -SS-), beta purothionin (same), human secretory leukocyte protease inhibitor (107 AA; 8 -SS-), hen egg-white lysozyme (129 AA; 8 -SS-), T4 lysozyme (164 AA; no -SS-), human lysozyme, endothelin (16 AA; 2 -SS-), apamin (a bee venom)(12 AA; 2 -SS-), E. coli heat-stable enterotoxin (Guarino et al., Infection and Immunity (1989), 57(2)649-52.)(18 AA; 3 -SS-), the Cucurbita maxima trypsin inhibitor CMTI-III (26 AA; 3 -SS-)(or other related protease inhibitors found in the seeds of Cucurbita species), the third domain of ovomucoid (56 AA; 3 -SS-), alpha-conotoxin

(13 AA; 2 -SS-); mu-conotoxin (22 AA; 3 -SS-); omega-conotoxin (27 AA; 3 -SS-); the Conus King Kong mini-protein (same); and azurin (128 AA; Cu:Cys,His,His,Met).

Most of the PBDs derived from a PPBD according to the process of the present invention will have been derived by variegation at residues having side groups directed toward the solvent. Reidhaar-Olson and Sauer (1988) found that exposed residues can accept a wide range of amino acids, while buried residues are more limited in this regard. Surface mutations typically have only small effects on melting temperature of the PBD, but may reduce the stability of the PBD. Hence the chosen IPBD should have a high melting temperature (50°C acceptable, the higher the better; BPTI melts at 95°C.) and be stable over a wide pH range (8.0 to 3.0 acceptable; 11.0 to 2.0 preferred), so that the SBDs derived from the chosen IPBD by mutation and selection-through-binding will retain sufficient stability. Preferably, the substitutions in the IPBD yielding the various PBDs do not reduce the melting point of the domain below ≈40°C. Mutations may arise that increase the stability of SBDs relative to the IPBD, but the process of the present invention does not depend upon this occurring. Proteins containing covalent crosslinks, such as multiple disulfides, are usually sufficient stable. A protein having at least two disulfides and having at least 1 disulfide per every twenty residues may be presumed to be sufficiently stable.

The binding protein initially used as a parental protein for variegation (IPBD) will preferably have less than 200 residues, more preferably less than 80 residues, and still more preferably less than 60 residues (not counting residues corresponding to the outer surface protein of the genetic package). Preferably, the IPBD is no larger than necessary because small SBDs (for example, less than 30 amino acids) can be chemically synthesized and because it is easier to arrange restriction sites in smaller amino-acid sequences. For PBDs smaller than about 40 residues, an added advantage is that the entire variegated pbd gene can

be synthesized in one piece. In that case, we need arrange only suitable restriction sites in the osp gene. A smaller protein minimizes the metabolic strain on the GP or the host of the GP. The IPBD must also be large enough to have acceptable binding affinity and specificity. For an IPBD lacking covalent crosslinks, such as disulfide bonds, the IPBD is preferably at least 40 residues; it may be as small as six residues if it contains a crosslink. These small, crosslinked IPBDs, known as "mini-proteins", are discussed in more detail later in this section.

A polypeptide is a polymer composed of a single chain of the same or different amino acids joined by peptide bonds. Linear peptides can take up a very large number of different conformations through internal rotations about the main chain single bonds of each α carbon. These rotations are hindered to varying degrees by side groups, with glycine interfering the least, and valine, isoleucine and, especially, proline, the most. A polypeptide of 20 residues may have 10^{20} different conformations which it may assume by various internal rotations.

Proteins are polypeptides which, as a result of stabilizing interactions between amino acids that are not in adjacent positions in the chain, have folded into a well-defined conformation. This folding is usually essential to their biological activity.

For polypeptides of 40-60 residues or longer, noncovalent forces such as hydrogen bonds, salt bridges, and hydrophobic "interactions" are sufficient to stabilize a particular folding or conformation. The polypeptide's constituent segments are held to more or less that conformation unless it is perturbed by a denaturant such as rising temperature or decreasing pH, whereupon the polypeptide unfolds or "melts". The smaller the peptide, the more likely it is that its conformation will be determined by the environment. If a small unconstrained peptide has biological activity, the peptide ligand will be in essence a random coil until it comes into proximity with

its receptor. The receptor accepts the peptide only in one or a few conformations because alternative conformations are disfavored by unfavorable van der Waals and other non-covalent interactions.

Mini-Proteins are small polypeptides (usually less than about 60 residues) which, while too small to have a stable conformation as a result of noncovalent forces alone, are covalently crosslinked (e.g., by disulfide bonds) into a stable conformation and hence have biological activities more typical of larger protein molecules than of unconstrained polypeptides of comparable size. Mini-proteins form a preferred class of initial parental binding proteins.

When mini-proteins are variegated, the residues which are covalently crosslinked in the parental molecule are left unchanged, thereby stabilizing the conformation. For example, in the variegation of a disulfide bonded mini-protein, certain cysteines are invariant so that under the conditions of expression and display, covalent crosslinks (e.g., disulfide bonds between one or more pairs of cysteines) form, and substantially constrain the conformation which may be adopted by the hypervariable linearly intermediate amino acids. In other words, a constraining scaffolding is engineered into polypeptides which are otherwise extensively randomized.

Once a mini-protein of desired binding characteristics is characterized, it may be produced, not only by recombinant DNA techniques, but also by nonbiological synthetic methods.

For the purpose of the appended claims, a mini-protein has between about eight and about sixty residues. However, it will be understood that a chimeric surface protein presenting a mini-protein as a domain will normally have more than sixty residues. Polypeptides containing intrachain disulfide bonds may be characterized as cyclic in nature, since a closed circle of covalently bonded atoms is defined by the two cysteines, the intermediate amino acid

residues, their peptidyl bonds, and the disulfide bond. The terms "cycle", "span" and "segment" will be used to define certain structural features of the polypeptides. An intrachain disulfide bridge connecting amino acids 3 and 8 of a 16 residue polypeptide will be said herein to have a cycle of 6 and a span of 4. If amino acids 4 and 12 are also disulfide bonded, then they form a second cycle of 9 with a span of 7. Together, the four cysteines divide the polypeptide into four intercysteine segments (1-2, 5-7, 9-11, and 13-16). (Note that there is no segment between Cys3 and Cys4.)

The connectivity pattern of a crosslinked mini-protein is a simple description of the relative location of the termini of the crosslinks. For example, for a mini-protein with two disulfide bonds, the connectivity pattern "1-3, 2-4" means that the first crosslinked cysteine is disulfide bonded to the third crosslinked cysteine (in the primary sequence), and the second to the fourth.

The variegated disulfide-bonded mini-proteins of the present invention fall into several classes.

Class I mini-proteins are those featuring a single pair of cysteines capable of interacting to form a disulfide bond, said bond having a span of no more than nine residues. This disulfide bridge preferably has a span of at least two residues; this is a function of the geometry of the disulfide bond. When the spacing is two or three residues, one residue is preferably glycine in order to reduce the strain on the bridged residues. The upper limit on spacing is less precise, however, in general, the greater the spacing, the less the constraint on conformation imposed on the linearly intermediate amino acid residues by the disulfide bond.

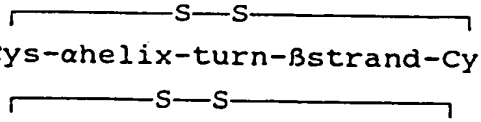
The main chain of such a peptide has very little freedom, but is not stressed. The free energy released when the disulfide forms exceeds the free energy lost by the main-chain when locked into a conformation that brings the cysteines together. Having lost the rree energy of

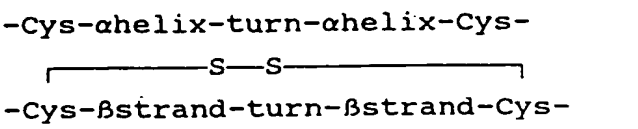
disulfide formation, the proximal ends of the side groups are held in more or less fixed relation to each other. When binding to a target, the domain does not need to expend free energy getting into the correct conformation. The domain can not jump into some other conformation and bind a non-target.

A disulfide bridge with a span of 4 or 5 is especially preferred. If the span is increased to 6, the constraining influence is reduced. In this case, we prefer that at least one of the enclosed residues be an amino acid that imposes restrictions on the main-chain geometry. Proline imposes the most restriction. Valine and isoleucine restrict the main chain to a lesser extent. The preferred position for this constraining non-cysteine residue is adjacent to one of the invariant cysteines, however, it may be one of the other bridged residues. If the span is seven, we prefer to include two amino acids that limit main-chain conformation. These amino acids could be at any of the seven positions, but are preferably the two bridged residues that are immediately adjacent to the cysteines. If the span is eight or nine, additional constraining amino acids may be provided.

The disulfide bond of a class I mini-proteins is exposed to solvent. Thus, one should avoid exposing the variegated population of GPs that display class I mini-proteins to reagents that rupture disulfides; Creighton names several such reagents (CREI88).

Class II mini-proteins are those featuring a single disulfide bond having a span of greater than nine amino acids. The bridged amino acids form secondary structures which help to stabilize their conformation. Preferably, these intermediate amino acids form hairpin supersecondary structures such as those schematized below:





Secondary structures are stabilized by hydrogen bonds between amide nitrogen and carbonyl groups, by interactions between charged side groups and helix dipoles, and by van der Waals contacts. One abundant secondary structure in proteins is the α-helix. The α helix has 3.6 residues per turn, a 1.5 Å rise per residue, and a helical radius of 2.3 Å. All observed α-helices are right-handed. The torsion angles ϕ (-57°) and U¦ (-47°) are favorable for most residues, and the hydrogen bond between the backbone carbonyl oxygen of each residue and the backbone NH of the fourth residue along the chain is 2.86 Å long (nearly the optimal distance) and virtually straight. Since the hydrogen bonds all point in the same direction, the α helix has a considerable dipole moment (carboxy terminus negative).

The ß strand may be considered an elongated helix with 2.3 residues per turn, a translation of 3.3 Å per residue, and a helical radius of 1.0 Å. Alone, a ß strand forms no main-chain hydrogen bonds. Most commonly, ß strands are found in twisted (rather than planar) parallel, antiparallel, or mixed parallel/antiparallel sheets.

A peptide chain can form a sharp reverse turn. A reverse turn may be accomplished with as few as four amino acids. Reverse turns are very abundant, comprising a quarter of all residues in globular proteins. In proteins, reverse turns commonly connect ß strands to form ß sheets, but may also form other connections. A peptide can also form other turns that are less sharp.

Based on studies of known proteins, one may calculate the propensity of a particular residue, or of a particular dipeptide or tripeptide, to be found in an α helix, ß strand or reverse turn. The normalized frequencies of occurrence of the amino acid residues in these secondary structures is

given in Table 6-4 of CREI84. For a more detailed treatment on the prediction of secondary structure from the amino acid sequence, see Chapter 6 of SCHU79.

In designing a suitable hairpin structure, one may copy an actual structure from a protein whose three-dimensional conformation is known, design the structure using frequency data, or combine the two approaches. Preferably, one or more actual structures are used as a model, and the frequency data is used to determine which mutations can be made without disrupting the structure.

Preferably, no more than three amino acids lie between the cysteine and the beginning or end of the α helix or ß strand.

More complex structures (such as a double hairpin) are also possible.

Class III mini-proteins are those featuring a plurality of disulfide bonds. They optionally may also feature secondary structures such as those discussed above with regard to Class II mini-proteins. Since the number of possible disulfide bond topologies increases rapidly with the number of bonds (two bonds, three topologies; three bonds, 15 topologies; four bonds, 105 topologies) the number of disulfide bonds preferably does not exceed four. With two or more disulfide bonds, the disulfide bridge spans preferably do not exceed 50, and the largest intercysteine chain segment preferably does not exceed 20.

Naturally occurring class III mini-proteins, such as heat-stable enterotoxin ST-Ia frequently have pairs of cysteines that are adjacent in the amino-acid sequence. Adjacent cysteines are very unlikely to form an intramolecular disulfide and cysteines separated by a single amino acids form an intramolecular disulfide with difficulty and only for certain intervening amino acids. Thus, clustering cysteines within the amino-acid sequence reduces the number of realizable disulfide bonding schemes. We utilize such clustering in the class III mini-protein disclosed herein.

Metal Finger Mini-Proteins. The mini-proteins of the present invention are not limited to those crosslinked by disulfide bonds. Another important class of mini-proteins are analogues of finger proteins. Finger proteins are characterized by finger structures in which a metal ion is coordinated by two Cys and two His residues, forming a tetrahedral arrangement around it. The metal ion is most often zinc(II), but may be iron, copper, cobalt, etc. The "finger" has the consensus sequence (Phe or Tyr)-(1 AA)-Cys-(2-4 AAs)-Cys-(3 AAs)-Phe-(5 AAs)-Leu-(2 AAs)-His-(3 AAs)-His-(5 AAs). While finger proteins typically contain many repeats of the finger motif, it is known that a single finger will fold in the presence of zinc ions. There is some dispute as to whether two fingers are necessary for binding to DNA. The present invention encompasses mini-proteins with either one or two fingers. It is to be understood that the target need not be a nucleic acid.

For a mini-protein that lacks α helices and ß strands, one will, in any given round of mutation, preferably variegate each of 4-6 non-cysteine codons so that they each encode at least eight of the 20 possible amino acids. The variegation at each codon could be customized to that position. Preferably, cysteine is not one of the potential substitutions, though it is not excluded.

When the mini-protein is a metal finger protein, in a typical variegation strategy, the two Cys and two His residues, and optionally also the aforementioned Phe/Tyr, Phe and Leu residues, are held invariant and a plurality (usually 5-10) of the other residues are varied.

When the mini-protein is of the type featuring one or more α helices and ß strands, the set of potential amino acid modifications at any given position is picked to favor those which are less likely to disrupt the secondary structure at that position. Since the number of possibilities at each variable amino acid is more limited, the total number of variable amino acids may be greater without altering the sampling efficiency of the selection process.

For the last-mentioned class of mini-proteins, as well as domains other than mini-proteins, preferably not more than 20 and more preferably 5-10 codons will be variegated. However, if diffuse mutagenesis is employed, the number of codons which are variegated can be higher.

The decision as to which residues to modify is eased by knowledge of which residues lie on the surface of the domain and which are buried in the interior.

We now proceed to discuss a few of the preferred IPBPs. Bovine pancreatic trypsin inhibitor (also known as aprotonin) is preferred because its structure is well known and the protein's scaffold can tolerate many changes on the surface. WO90/02809, which is included by reference, contains a detailed hypothetical example of 1) variegation of a phage-displayed protein (BPTI in that example) and 2) selection of phage that display a protein having high affinity for a predetermined target.

Example 1 of Ser. No. 240,160 (WO90/02809) describes a strategy for the initial extensive mutagenesis ("variegation") of BPTI and the same strategy may be adopted here. However, the present invention is not limited to use of any particular variegation pattern, though the limitations of the screening system should be kept in mind.

In another preferred embodiment, the IPBP is CMTI-I or CMTI-III. CMTI-I and CMTI-III are members of the squash family of serine protease inhibitors, which also includes inhibitors from summer squash, zucchini, and cucumbers (Wieczorek et al., Biochem Biophys Res Comm (1985), 126(2)646-652; Otlewski et al., Biol Chém Hoppe-Seyler (1987), 368:1505-7; and Favel et al., Biochem Biophys Res Comm (1989), 162:79-82.). McWherter et al. (Biochemistry (1989), 28:5708-14) describe synthetic sequence-variants of the squash-seed protease inhibitors that have affinity for human leukocyte elastase and cathepsin G. Of course, any member of this family might be used.

CMTI-I is one of the smallest proteins known, comprising only 29 amino acids held in a fixed conformation by

three disulfide bonds. The structure has been studied by Bode and colleagues using both X-ray diffraction (Bode et al., FEBS Lett (1989), 242(2)285-92) and NMR (Holak et al., J Mol Biol (1989), 210:635-648 and Holak et al., J Mol Biol (Dec 5 1989), 210(3)649-54). CMTI-I is of ellipsoidal shape; it lacks helices or ß-sheets, but consists of turns and connecting short polypeptide stretches. The disulfide pairing is Cys3-Cys20, Cys10-Cys22 and Cys16-Cys28. In the CMTI-I:trypsin complex studied by Bode et al., 13 of the 29 inhibitor residues are in direct contact with trypsin; most of them are in the primary binding segment Val2(P4)-Glu9 (P4') which contains the reactive site bond Arg5(P1)-Ile6 and is in a conformation observed also for other serine proteinase inhibitors.

CMTI-I has a K_i for trypsin of $\approx 1.5 \cdot 10^{-12}$ M. McWherter et al. suggested substitution of "moderately bulky hydrophobic groups" at P1 to confer HLE specificity. They found that a wider set of residues (VAL, ILE, LEU, ALA, PHE, MET, and GLY) gave detectable binding to HLE. For cathepsin G, they expected bulky (especially aromatic) side groups to be strongly preferred. They found that PHE, LEU, MET, and ALA were functional by their criteria; they did not test TRP, TYR, or HIS. (Note that ALA has the second smallest side group available.) A preferred initial variegation strategy using CMTI-I as the parental protein would be to vary some or all of the residues ARG_1, VAL_2, PRO_4, ARG_5, ILE_6, LEU_7, MET_8, GLU_9, LYS_{11}, HIS_{25}, GLY_{26}, TYR_{27}, and GLY_{29}. If the target were HNE, for example, one could synthesize DNA embodying the following possibilities:

Parental	vg Codon	Allowed amino acids	#AA seqs/ #DNA seqs
ARG_1	VNT	RSLPHITNVADG	12/12
VAL_2	NWT	VILFYHND	8/8
PRO_4	VYT	PLTIAV	6/6
ARG_5	VNT	RSLPHITNVADG	12/12
ILE_6	NNK	all 20	20/31
LEU_7	VWG	LQMKVE	6/6
TYR_{27}	NAS	YHQNKDE.	7/8

This allows about $5.81 \cdot 10^6$ amino-acid sequences encoded by about $1.03 \cdot 10^7$ DNA sequences. A library comprising $5.0 \cdot 10^7$ independent transformants would give ≈99% of the possible sequences. Other variegation schemes could also be used.

The present invention is not limited to the use of a known protein (including mini-proteins) as a parental protein. To obtain a library of binding domains that are conformationally constrained by a single disulfide, we insert DNA coding for one of the following families of mini-proteins into the gene coding for a suitable OSP.

$$X_1\text{-}X_2\text{-}C\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}C\text{-}X_7\text{-}X_8 \qquad \text{TN4}$$

$$X_1\text{-}X_2\text{-}C\text{-}X_3\text{-}X_4\text{-}X_5\text{-}X_6\text{-}X_7\text{-}C\text{-}X_8\text{-}X_9 \qquad \text{TN5}$$

Where ⌊——⌋ indicates disulfide bonding. Conformationally restrained peptides having six or more amino-acid residues between the cysteines may be used, but the effect of the restraint diminishes as the loop becomes larger. Disulfides normally do not form between cysteines that are consecutive on the polypeptide chain. One or more of the residues indicated above as X_n will be varied extensively to obtain novel binding. In TN4, there may be one or more amino acids that precede X_1 or follow X_8, however, these additional residues will not be significantly constrained by the diagrammed disulfide bridge, and it is less advantageous to vary these remote, unbridged residues. Similarly, in TN5, there may be one or more amino acids that precede X_1 or follow X_9. The last X residue is connected to the OSP of the genetic package.

In TN4, X_1, X_2, X_3, X_4, X_5, X_6, X_7, and X_8 can be varied independently; i.e. a different scheme of variegation could be used at each position. X_1 and X_8 are the least constrained residues and may be varied less than other positions.

X_1 and X_8 can be, for example, one of the amino acids [E, K, T, and A]; this set of amino acids is preferred because: a) the possibility of positively charged, negatively charged, and neutral amino acids is provided, b) these amino acids can be provided in 1:1:1:1 ratio via the codon RMG (R = equimolar A and G, M = equimolar A and C), and c) these amino acids allow proper processing by signal peptidases.

One option for variegation of X_2, X_3, X_4, X_5, X_6, and X_7 is to vary all of these in the same way. For example, each of X_2, X_3, X_4, X_5, X_6, and X_7 can be chosen from the set [F, S, Y, C, L, P, H, R, I, T, N, V, A, D, and G] which is encoded by the mixed codon NNT. NNT encodes 15 different amino acids and only 16 DNA sequences. Thus, there are $1.139 \cdot 10^7$ amino-acid sequences, no stops, and only $1.678 \cdot 10^7$ DNA sequences. A library of 10^8 independent transformants will contain 99% of all possible sequences. The NNK library contains $6.4 \cdot 10^7$ sequences, but complete sampling requires a much larger number of independent transformants.

The potential binding protein usually is produced as a domain of a chimeric protein incorporating a suitable outer surface protein of the genetic package. The preferred genetic package is a filamentous phage, such as M13 or f1. The preferred outer surface proteins are the gene III protein of M13 and the gene VIII protein of M13.

For screening, any assay for the determination of binding to proline-rich proteins which is not lethal to the phages or cells used to carry the candidate binding proteins may be used. A preferred screening procedure is described below but the invention is not limited to use of filamentous phage or the specific chromatographic materials used:

A. The phage-displayed binding protein population is initially passed over albumin-blocked HAP beads without PRPs bound to them, and the phage bearing proteins which do not bind, the "eluate phage", are collected. This procedure eliminates phage that display proteins which bind strongly to the

HAP alone.

B. The appropriate PRPs are bound to HAP as previously described (Gibbons and Hay, 1988) to form an "experimental pellicle".

C. The eluate phage are then passed over the experimental pellicle and those which bind (those carrying "PRP binding proteins") are collected by desorbing them from the experimental pellicle by varying temperature, the concentration of salt, urea, guanidinium chloride, or other suitable eluting agent, or (with limitations) pH.

D. These PRP binding proteins, which are associated with vectors containing their genes, are then amplified to provide sufficient material for further screening.

E. Optional steps to increase stringency of selection--

1. Return to step C with selected phage population obtained in Step D, or

2. A large excess of plaque bacteria, B. gingivalis, A. viscosis, etc., are bound to the experimental pellicle to saturate the PRPs, and the pellicle is incubated with the amplified pool of PRP binding proteins for a time long enough for the binding proteins to displace the plaque bacteria. Optionally, the HAP beads are agitated or brushed during the incubation in a manner that simulates tooth brushing. The proteins which bind to the experimental pellicle under these conditions should be mainly those which bind tightly enough to displace the plaque bacteria. These "high affinity" PRP binding proteins are then desorbed from the experimental pellicle as described in step C.

The high affinity PRP binding proteins are amplified using recombinant DNA techniques. Preferably, the genes encoding these PRP binding proteins are isolated from the

genetic package and recloned into a high efficiency expression system.

When fractionating a variegated population of phage on experimental pellicles to obtain a protein that binds to PRP-HAP, we must exercise care not to wash the PRPs off the HAP. For example, low pH dissolves HAP and is an inappropriate eluant for this purpose. Urea or guanidinium hydrochloride, buffered to pH near 7, are appropriate.

Each monomer of the major coat protein (gene VIII protein) of fd and f1 carries one more acidic (negative) residue than does each monomer of the coat protein of M13. M13 phage are strongly negatively charged and phage f1 and fd are even more strongly charged. Because the surface of hydroxyapatite (HAP) is positively charged, M13 adheres strongly to HAP.

Initial experiments were conducted with PRP-1 bound to HAP beads (Gallard-Schlessinger). A derivative of M13mp18 phage were applied in "saliva" buffer: 50 mM KCl, 1mM KH_2PO_4/K_2HPO_4, 1mM $CaCl_2$, 0.1 mM MgCl, pH 6.0. Under these conditions, about 34% of phage stick to HAP beads and can be eluted with 3 M guanidine HCl. A large fraction of the phage can be washed away with higher salt: saliva buffer brought up to 150 mM in KCl leaves only 0.1% of the input phage, while 250mM KCl saliva buffer leaves only .09%. Higher salt can be used so long as one does not wash the PRP-1 off the HAP; 150 mM KCl is known not to remove PRP-1.

Another approach to fractionating a population of potential PRP-1 binding proteins is to support PRP-1 on a different matrix that does not bind wild-type phage non-specifically. A. viscosis binds PRP-1 that is covalently linked to agarose, though not so well as the bacteria binds PRP-1 on HAP. Thus, PRP-1 immobilized on agarose is suitable for fractionating a population of phage that bear potential PRP-binding proteins.

Another approach to fractionating a population of potential PRP-1 binding proteins is to display them on bacterial cells, as disclosed in WO90/02809. HAP beads that

are coated with PRP-1 may be used to fractionate a variegated (extensive mutagenized) population of bacterial cells that display potential PRP-binding proteins. A suitable combination of host, OSP, and parental protein for this embodiment is E. coli, OmpF, and CMTI-III.

OmpF of E. coli is a very abundant OSP, $\geq 10^4$ copies-/cell. Pages et al. (Pages et al., Biochimie (1990), 72:169-76.) have published a model of OmpF indicating seven surface-exposed segments. Fusion of an initial-potential-binding-domain (ipbd) gene fragment, either as an insert or to replace the 3' part of ompF, in one of the indicated regions is likely to produce a functional ompF::ipbd gene the expression of which leads to display of IPBD on the cell surface. In particular, fusion at about codon 111, 177, 217, or 245 should lead to a functional ompF::ipbd gene. Concerning OmpF, see also Reid et al., J Biol Chem (1988), 263(16)7753-9; Pages and Bolla, Eur J Biochem (1988), 176(3)655-60; Benson et al., J Mol Biol (1988) 203(4)961-70; Tommassen et al., Mol gen Genet (1982), 185:105-110; and Sodergren et al., J Bacteriol (1985), 162(3)1047-1053; all of which are incorporated by reference.

The method is not limited to use of any particular matrix or coupling procedure. Matrices known in the art include: Hydroxyapatite (for PRPs), activated cross-linked agarose (such as Affi-Gel 10 and Affi-Gel 15 from BioRad (Richmond, CA) or Reacti-Gel 6X form Pierce Chemical Company (Rockford, IL), activated Trisacryl from Pierce Chemical Company, activated TSK HW-65F from Pierce Chemical Company, polystyrene, and silica (e.g., SelectiSpher-10 Activated Tresyl Silica).

Proline-rich proteins may be purified from saliva by the methods set forth in Gibbons and Hay (1988b) and in the works cited therein. The sequences of five PRPs are known and these proteins could be expressed in transformed cells. PRPs contain phosphoserine, so that expression in eukaryotic cells possessing appropriate serine kinase is preferred.

Statherin may be obtained from saliva by the methods

set forth in Gibbons and Hay (1989) or Schlesinger, et al. (1977) and in the works cited therein. The amino acid sequence of statherin is known and this polypeptide could be chemically synthesized.

The target protein (e.g., PRP or statherin) may be coupled to the matrix by:

(a) reagents that react with free amine groups, such as imidazolyl carbamates, N-hydroxysuccinimide esters, or EDAC (1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride, see BioRad March 1989 catalogue, p. 67);

(b) reagents that react with carboxylic acid groups,such as EDAC;

(c) reagents that react with thiol groups, such as Affi-Gel 401 from BioRad or alkyl iodides (e.g., SulfoLink Coupling Gel from Pierce); and

(d) reagents that react with oxidized carbohydrates, such as a hydrazide (CarboLink Hydrazide from Pierce).

For additional coupling methods, see Mossbach, et al., Meth. Enzymol., 44: 3-7 and 53-65 (1976), incorporated by reference herein. While the matrix is conveniently provided in bead form, other forms suitable for affinity separation may be employed.

Note that PRPs that are free in solution do not bind bacterial adhesins, while PRPs bound to HAP on the surface of a tooth do bind bacterial adhesion. Thus, to develop a protein that binds to PRPs on a tooth surface, we prefer HAP as a support matrix. Any other support that causes the PRP to become active with respect to binding of bacterial adhesins could also be used.

Individual high affinity PRP binding proteins will be selected and further characterized for their ability to displace a variety of representative plaque bacteria from HAP. The kinetics of displacement will be studied using radiolabelled plaque bacteria. From these studies, one or more "final" proteins will be selected.

One question which arises is whether the PRP binding proteins can actually displace most of the plaque bacteria in the short time the dental composition is used. A related question is whether the expected small amounts of PRP binding protein in the dental composition are sufficient to bind the large number of PRPs in a typical mouth.

We calculate that it would take approximately 10^{-5} percent, a very small amount, of PRP binding protein in a mouthwash to saturate the PRPs in the mouth.

The number of PRP molecules per square micron in the pellicle is approximately 10^4 (R. Gibbons and D. Hay, personal communication). The total tooth area in a typical mouth is about 64 cm^2. Thus, the number of PRP molecules in the pellicle in a whole mouth is given by

$$(10^8 cm^2/micron^2) \times 10^4 \times 64 = 6.4 \times 10^{13}$$

Thus, it would take 6.4×10^{13} binding proteins to bind to all the PRPs in the mouth. Since a typical binding protein would be expected to have about 100 amino acids, each having a molecular weight of about 120 daltons, the grams of binding protein required is

$$120 \times 100 \times 6.4 \times 10^{13} / 6.023 \times 10^{23} = 1.3 \times 10^{-6} \text{ grams.}$$

This small number of grams could readily be included in a typical mouthwash rinse of 20 milliliters.

We have also made a calculation which indicates that during a typical mouthwash rinse of 5-20 seconds there is enough time for the binding proteins to find and bind to the PRPs.

The reaction whose kinetics we wish to predict is the following:

$$B + P \xrightarrow{k} BP$$

where B signifies binding protein, P signifies PRP receptor, and k is the rate constant for the reaction.

For simple binding to a free receptor (i.e., one to

which no bacterial adhesins are bound) the standard second order kinetic equation can be solved assuming binding protein is present in great excess to give

$$B = [-\ln(1-F)]/kt$$

where B is the binding protein concentration in the mouth required to bind a fraction F of PRP receptors in time t, given that the rate constant for the reaction is k.

Typically protein-protein reactions proceed with a rate constant of greater than $K=10^6$ liters/mole. To be conservative we will choose a much slower rate constant of $k=10^5$ liters/mole. We assume that our goal is to bind F=0.999 (99.9%) of the PRP receptors in a gargle-time of t=5 sec.

With these assumptions, the equation above yields for the concentration of required binding protein

$$B = 1.38 \times 10^{-5} \text{ moles/liter.}$$

Our next task is to find the amount of binding protein in a typical mouthwash required to give the above mole per liter concentration. First, we assume the whole mouth volume in which the mouthwash is being sloshed is V=200 ml. Thus, the number of moles, N, of binding protein needed in the gargle is

$$N = 1.38\times10^{-5} \times 200/1000 = 2.76\times10^{-6} \text{ moles}$$

Given a typical molecular weight for binding protein of 1.2×10^4 daltons, the number of grams of binding protein required is

$$1.2\times10^4 \text{ gams/mole} \times 2.76\times10^{-6} = 0.033 \text{ grams}$$

This 0.033 grams of binding protein must be present in about 20 mls of mouthwash used in the gargle. Therefore, its percentage concentration in the mouthwash is

$$0.033\times100/20 = 0.165\%,$$

a reasonable percentage to be included in a mouthwash.

Again this calculation is approximate but it is also very conservative in that the reaction of a single receptor with a single binding protein could be faster, and in practice, less than the assumed 99.9% of the PRP receptors could be bound to be effective.

The calculations do not take into account that the PRPs, in addition to binding to receptors, also replace bacteria already bound to the receptors. Furthermore, each bacterium is probably bound to many receptors. However, given that bacterial adhesin binding to receptors is thought to be weak (Karlsson, 1989) so that displacing these weakly bound adhesins ought to be fairly rapid, the first-order effects described should be dominant.

Furthermore, the approach of using receptor binding analogs to compete with bacterial adhesins has been successful with urinary tract infections (Gibbons, 1990 and see Appendix III; Aronson, et al., 1979; Svanborg-Eden, et al., 1982). Also, cloned S. sanguis adhesin has been shown to desorb S. sanguis from saliva-coated hydroxyapatite (Ganeshkumar, et al., 1988).

The result of our approximate but conservative analysis indicates that a typical bottle of mouthwash or tube of toothpaste would need to contain only about .2% anti-plaque binding protein to achieve significant efficacy.

PRP-binding proteins can be tested for toxicity and efficacy in standard ways. As a wide variety of proteins are found in the human diet, toxicity of these agents is likely to be very low or undetectable. In particular, two parental proteins here contemplated, BPTI (found in almost all beef organs and flesh) and CMTI-III (found in pumpkin seeds) are part of the normal human diet.

Efficacy can be established by treating patients in accord with a set protocol for a short period of time and then comparing plaque build-up between treated and control teeth. Suppose that we identify protein XYZ as a PRP-binding protein that shows efficacy in vitro. For example, in a group of thirty subjects, one could treat right-side

teeth for two weeks with a 10 ppm (parts-per-million) solution of protein XYZ. During this time, the left side is treated with a placebo, perhaps bovine serum albumin or the protein that is parent to XYZ. After two weeks, we exchange the sides treated in all or some of the subjects. Comparison of the amount of plaque on treated and untreated teeth gives an indication of the efficacy of XYZ (or any other PRP-binding protein).

Glucosyltransferase (GTF) Inhibitors

GTF is the enzyme, found on the surface of streptococci such as S. mutans, S. sanguis, S. mitis and S. sobrinus and found free in saliva, which polymerizes sugar monomers to form the web or matrix which prevents plaque bacteria from washing away from the teeth (Rolla, 1989). Inhibitors of GTF are known to exist (Takada, et al., 1985; Koga, et al., 1982; Endo, 1983), and one of these was shown to inhibit plaque formation in vitro and caries in rats (Takada, et al., 1985). Moreover, one of the inhibitors, mutastein, is a protein (Endo, 1983). Thus, the present invention contemplates making proteins which bind tightly to the active site of GTF and thereby act as inhibitors to the enzyme and also as plaque-reducing agents.

Free GTF in the saliva has been shown to adsorb to the pellicle, and in the adsorbed state can synthesize glucan (i.e., the polysacharride matrix). Thus, in a different embodiment, binding proteins may be made which bind to the pellicle at the site of adsorption to prevent binding of the enzyme.

To remain effective in the mouth, the GTF inhibitors should be tethered. We envision that a "polymer" of GTF inhibitors would be constructed by expressing fused mutant genes each encoding a GTF-binding peptide. This polymer would consist of several GTF inhibitor molecules (the "units") tandem to each other with a short amino acid spacer or linker sequence connecting them. The end unit of the polymer would then bind to a GTF molecule on the surface of

S. mutans. thereby tethering the polymer at the site where it is needed. The other inhibitor units in the polymer would then bind to other GTF molecules as they are made by the S. mutans and as the S. mutans cells divide.

This polymer of tandemly-linked GTF inhibitors can be made by genetic engineering using tandemly-repeated genes. Such a genetically engineered construction would allow polymer manufacture in one step in an appropriate host organism. The genetic constructions leading to this polymer of GTF inhibitors is illustrated schematically in Fig. 7, along with an illustration of how they should work in the mouth.

The process of identifying the desired GTF inhibitors differs from that described for making PRP-binding protein embodiment only in the use of a different screening procedure. For example, GTF may be supported on a wider variety of supports than is appropriate to PRPs. It is preferred that GTF be covalently attached to the matrix; for example, GTF could be attached to Reacti-Gel (Pierce Chemical Company). Fractionation of a variegated population of phage that display potential GTF-inhibitor proteins may be accomplished with a wider variety of eluants than is appropriate for elution of PRP -HAP matrices; a gradient of decreasing pH is appropriate for GTF covalently attached to agarose (or other matrix). GTF may be obtained by the methods set forth in Mukasa et al. (1989) and the works cited therein.

Any method of screening for GTF-inhibitory activity which is not fatal to the genetic packages displaying the muteins is acceptable, but the following procedure is preferred:

A. The population of phage (or cells or spores) that display candidate GTF-binding proteins are passed over a control matrix of agarose beads, and the phage which do not bind, the "eluate phage", are collected. This procedure eliminates proteins which bind to the cyanogen bromide activated

agarose.

B. Prepare GTF bound to the imidazolyl carbamate- activated agarose bead matrix, and verify GTF activity in this bound state.

C. The eluate phage are then passed over the GTF-beads and the phage that display GTF-binding are collected by desorbing them from the beads by varying pH, temperature, salt, etc.

D. The phage that display GTF-binding proteins are then amplified to make amounts needed for subsequent steps.

E. The genes that encode putative GTF-binding proteins are transferred, by standard recombinant DNA techniques, to a production strain so that the candidate GTF-binding proteins can be produced as free proteins. We could transfer the DNA that codes for the GTF- binding domain into a suitable vector, or we could delete the phage-associated portion of the fusion gene that comprises the parental GTF- binding domain and a phage outer-surface protein (viz. gene III or VIII).

F. The soluble GTF-binding candidates will be tested individually for their ability to block glucan synthesis. (So that colonies which inhibit glucan synthesis can be readily picked, it may be useful here to employ a colorimetric assay for glucan synthesis.)

This embodiment should not be construed to be limited to the particular "genetic package" support matrix, coupling means, or desorption means described above.

Tethered Glucose Oxidase or Amyloglucosidase

Ideally, an anti-plaque agent should show significant efficacy when applied via the normal material usage and frequency of mouthwashing or brushing. As noted above, the effectiveness of many potential anti-plaque agents, such as enzymes, is roughly proportional to the time-of-residence in

the mouth. To achieve efficacious time-of-residence in the mouth under normal patterns of mouthwash or dentifrice use, we contemplate developing protein tethers designed to bind to the pellicle or plaque, and which also have an anti-plaque agent attached to them. Depending on the anti-plaque agent and the approach to plaque reduction, we can conceive of several types of tethers.

a. Leashes. These are tethers which are designed to bind to the pellicle or plaque at a specific location and have attached to them an anti-plaque agent which is free to move in a region adjoining the site of tethering. The leashes would be long protein chains, one end of which is attached to a binding protein directed to the desired location in the mouth and the other end is attached to a anti-plaque agent. Using recombinant DNA procedures, this entire unit consisting of a binding protein, leash, and anti-plaque agent can be made in one step if the anti-plaque agent is also a protein such as an enzyme.

In Fig. 5a, an example of a leash tether is illustrated. The figure shows one end of the leash bound to a RPR receptor and carrying an enzyme on the other hand.

b. Time-release tethers. These tethers do not need to have protein chains as long as leashes, but instead are designed to hydrolyze at rates consistent with most effective release of an anti-plaque agent. An example of a time-release tether releasing an anti-plaque agent is illustrated in Fig. 5b.

Some targets for tethers are the tooth surface (hydroxyapatite), bacterial receptors on PRP proteins, surfaces of pathogenic bacteria, other plaque components, or sites of caries or periodontal disease damage.

In addition, tethers can be made from natural human proteins which bind to oral surfaces such as PRP proteins and human albumin. In this way, immune responses could be minimized.

There is much to be said for the development of a

"universal tether", such as a PRP-binding protein. Once such a tether is made available, it can be used to tether most potentially efficacious proteins and non-protein molecules to the pellicle. Using a tether, a variety of mouthwash agents could be tested rapidly.

The potential efficacy and mode of action of glucoseoxidase and amyloglucosidase have already been discussed. In a preferred embodiment we would tether these enzymes to the pellicle using the PRP proteins as the site of tether attachment.

We previously described a plan to develop PRP-binding proteins. These binding proteins would be used as tethers for glucose-oxidase and amyloglucosidase. The enzymes can be covalently bound to the tether by a number of chemical methods used by protein chemists. Since glucose-oxidase and amyloglucosidase are already included in commercial dentifrices, they should be readily available. Therefore, once the tether is prepared the tethered version of the enzymes can be prepared quickly.

Tethering conditions must be chosen so that the enzyme activity is not significantly diminished. Methods of attaching an enzyme to a support or to a carrier protein are well known and are disclosed, e.g., in the BioRad catalogue, the Pierce Chemical Company catalogue, and numerous references therein.

Once a gene encoding a desired binding protein is identified, it may be operably linked to a promoter and cloned on a suitable vector into a host in which the promoter is functional. Bacillus subtilis, Escherichia coli and Saccharomyces cerevisiae are the preferred hosts.

Figure 8 shows a gene suitable for production of aprotinin-derived proteins in E. coli. A $LacI_q$ strain is preferred; suitable strains include XL1-blue (Stratagene, LaJolla, CA). The Tac promoter gives high levels of transcription when induced with IPTG; other promoters, such as wkw P_L, could be used. The Ribosome Binding Site (RBS) shown gives high levels of translation; other RBSs could be

used. The phoA signal sequence causes the protein to be secreted into the periplasm; other functional signal sequences could be used. Several stop codons are provided to avoid translation read- through. The transcription terminator prevents transcription read through, other terminator sequences could be used. Other host cells could be used. Methods in Enzymology, 185, reviews methods of expressing genes in a wide variety of cell types. The sequence of Figure 8 encodes native aprotonin. Muteins of aprotonin may be obtained by replacement, insertion or deletion of codons, as taught in Ser. No. 07/240,160 (WO90/02809).

Expression of heterologous genes in Bacillus subtilis is reviewed in Methods in Enzymology, 185, Section III comprising articles 17-20. Article 17 ("Expression of Heterologous Genes in Bacillus subtilis", pp. 199-201) is by Henner. Article 18 ("Regulated Promoter for High-Level Expression of Heterologous Genes in Bacillus subtilis", pp. 201-214) is by LeGrice. Nagarajan contributed article 19 ("System for Secretion of Heterologous Proteins in Bacillus subtilis", pp. 214-223). Henner wrote article 20 ("Inducible Expression of Regulatory Genes in Bacillus subtilis", pp. 223- 228). Suitable promoters for B. subtilis include P_{N25} (Stueber et al., EMBO J., 3:3143 (1984)), and $P_{N25/0}$ (LeGrice, Methods in Enzymology, 185:201 (1990)).

Methods in Enzymology, 185, Section IV (comprising articles 21-37) reviews expression of heterologous genes in yeast. Some suitable inducible promoters for expression in yeast are: GAL1 GAL7, and GAL10 (Mylin et al., Meth. Enzymol, 185:207-308). PGK is a suitable constitutive promoter (Kingsman et al., Meth. Enzymol, 185:329-341).

The anti-dental plaque agents of the present invention may be incorporated into any composition useful in the treatment of dental plaque in humans or animals. The composition may be a solid, liquid, gel or paste, and, without limitation, may take the form of a toothpaste,

dental cream, dental gel, tooth powder, liquid dentifrice, chewing gum, lozenge, ointment, chewable tablet, or mouthwash. The composition may comprise other anti-plaque agents as well as other ingredients which do not substantially interfere with the anti-plaque action of the aforementioned agents. The term "dental compositions" includes both compositions for use by patients, and compositions to be applied by dentists.

The anti-dental plaque agent may also be admixed with a foodstuff or beverage. The foodstuff may be, e.g., a breakfast cereal, bread, candy or ice cream. The beverage may be drinking water, milk, juice, or other soft drink, especially a sweetened drink.

Moreover, a dental cleaning appliance, such as a dental toothpick or dental floss, may be impregnated with a solution containing the agent.

The dental composition should have a pH practicable for use, preferably a pH of about 4 to about 10, and in particular about 7.

A toothpaste, cream or gel will usually be provided in a collapsible tube, typically aluminum, lined lead or plastic, or other squeeze pump or pressurized dispenser for metering out the contents.

The dental composition, especially a toothpaste (cream), tooth powder or dental gel, may contain abrasive agents. Particularly suitable abrasive agents include aluminum oxide, aluminum hydroxide, talc, pumice, dicalcium phosphate, calcium secondary phosphate dihydrate or anhydrate, calcium primary phosphate, calcium tertiary phosphate, calcium carbonate, calcium pyrophosphate, insoluble sodium metaphosphate, amorphous or crystal silica, aluminosilicate, magnesium tertiary phosphate, magnesium carbonate, calcium sulfate, titanium dioxide, resins, bentonite and the like, or mixtures thereof.

In a toothpaste, if an abrasive agent is provided it will usually constitute about 5% to about 95%, especially 10% - 50%, by weight of the composition.

A compatible binder or thickener may be used to adjust the viscosity of the dental composition. In particular, toothpastes (creams) and gels typically contain a natural or synthetic thickener or gelling agent. Orally acceptable thickeners include Irish moss, carboxymethyl cellulose, methyl cellulose, carboxymethyl hydroxyethyl cellulose, hydroxyethyl cellulose, sodium alginate, carrageenan, gum arabic, xanthan gum, tragacanth gum, karaya gum, polyvinyl alcohol, sodium polyacrylate, carboxyvinyl polymer, polyvinyl pyrolidone, colloidal silica, and the like, or mixtures thereof. In a toothpaste, the binder is typically blended in at a proportion of 0.1 to 12% by weight.

The composition may comprise a humectant, such as polyethylene glycol, ethylene glycol, sorbitol, glycerol, propylene glycol, 1,3-butylene glycol, xylitol, maltilol, lactitol, and the like, or mixtures thereof. The liquid vehicle, if any, may also include water. The humectant in a toothpaste is typically 10% - 80% by weight.

A compatible surface-active agent may be used to increase the prophylactic action and to disperse the anti-plaque agent throughout the oral activity. Preferably, it imparts detergent and foaming properties to the composition. This agent may be narionic, anionic, cationic, zwitterionic or amphoteric in character. Suitable surface-active agents include the soaps, such as the water-soluble salts of higher fatty acids or rosin acids, such as may be derived from fats, oils and waxes of natural origin, and the sulfated and sulfonated synthetic detergents, typically having 8 to 26 carbon atoms per molecule. Examples include water-soluble salts of higher fatty acid monoglyceride monosulfates, higher alkyl sulfates, alkyl aryl sulfonates, higher alkyl sulfoacetates, higher fatty acid esters of 1,2-dihydroxy propane sulfonates or isothionic acid, higher fatty acid amides of taurine, substantially saturated higher aliphatic acyl amides of lower aliphatic aminocarboxylic acid compounds, and the like, or mixtures thereof. Specific examples include sodium lauryl sulfate, sodium palmityl

sulfate and sodium myristyl sulfate, which are anionic surfactants, and lauryl diethanolamide, a nonionic surfactant. In a toothpaste, the surfactant is typically 0.1 to 7% by weight.

The compositions of this invention can be incorporated in lozenges, or in chewing gum or other products, e.g., by stirring into a warm gum base or coating the outer surface of a gum base, illustrative of which may be mentioned jelutone, rubber latex, vinylite resins, etc., desirably with conventional plasticizers or softeners, sugar or other sweeteners or carbohydrates such as glucose, sorbitol and the like.

The vehicle or carrier in a table or lozenge is typically a non-cariogenic solid water soluble polyhydric alcohol (polyol) such as mannitol, xylitol, sorbitol, maltitol, a hydrogenated starch hydrolysate, Lycasin, hydrogenated glucose, hydrogenated disaccharides, and hydrogenated polysaccharides, in an amount of about 90-98% by weight of the total composition. Solid salts such as sodium bicarbonate, sodium chloride, potassium bicarbonate or potassium chloride may totally or partially replace the polyol carrier.

Tableting lubricants, in minor amounts of about 0.1 to 5% by weight, may be incorporated into the tablet or lozenge formulation to facilitate the preparation of both the tablets or lozenges. Suitable lubricants include vegetable oils such as coconut oil, magnesium stearate, aluminum stearate, talc, starch and carbowax.

Lozenge formulations usually contain about 2% gum as barrier agent to provide a shiny surface as opposed to a tablet which has a smooth finish. Suitable non-cariogenic gums include Kappa carrageenan, carboxymethyl cellulose, hydroxyethyl cellulose. Gantrez, and the like.

The lozenge or tablet may optionally be coated with a coating material such as waxes, shellac, carboxymethyl cellulose, polyethylene/maleic anhydride co-polymer or Kappacarrageenan to further increase the time it takes the

tablet or lozenge to dissolve in the mouth.

The dental composition may contain any suitable flavoring or sweetening ingredients. Examples of suitable flavoring constituents may include, for instance, sodium methylsalicylate, and the flavoring oils, e.g., oils of spearmint, peppermint, wintergreen, sassafras, clove, sage, eucalyptus, marjoram, cinnamon, lemon, and organe, as well as other suitable flavors. Suitable sweetening agents include sucrose, lactose, maltose, sorbitol, sodium cyclamate, saccharin, asparatame, stevioside, neohesperidyl dihydrochalcone, glycyrrhizin, perillartine, p-methoxycinnamic aldehyde, etc. The flavoring and sweetening typically constitute 0.01% - 2% of the composition, but are not limited to this range.

The composition may further include coloring or whitening agents (e.g., titanium dioxide) or preservatives (e.g., sodium benzoate), or other anticaries or antiplaque agents.

A mouthwash may contain an effervescent granule, such as one containing tartaric acid and sodium hydrogen carbonate as effervescent agent.

The composition may also contain other anti-caries and anti-plaque agents, including enzymes such as various proteinases, lipases, phospholipases and carbohydrases (e.g., dextranase), invertase, and oxidoreductases (e.g., glucose oxidase), enzyme inhibitors such as certain dextrans and glucans, fluorine compounds such as sodium fluoride, stannous floride, potassium fluoride, and sodium monofluorophosphate, antibodies against surface antigens of plaque-forming bacteria, chlorophyll, (see Colman, U.S. 4,442,085 and Miyahara, U.S. 4,725,428), lectins (see Suddick, U.S. 4,217,341) and so forth.

For other suitable ingredients, see Accepted Dental Therapeutics.

For examples of how these various ingredients are combined into typical dentifrice compositions, see the references discussed above. Hoogendoorn, U.S. 4,178,362 is

worthy of note in that it provides specific toothpaste, tooth powder, mouthwash, chewing tablet, chewing gum and lozenge tablet composition, each employing glucose oxidase as the active ingredient. For other such comparative examples of compositions, see Ioka, U.S. 4,469,673, and Miyahara, U.S. 4,725,428. The anti-plaque agents of the instant invention may be substituted for Hoogendoorn's glucose oxidase, Iioka's dextranase or Miyahara's antibody, but of course, the present invention is not limited to particular combination or proportion of ingredients.

All references cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art or pertinent prior art.

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CLAIMS

1. Use of a binding protein which competes with a bacterial adhesin for a pellicular binding site in the manufacture of a composition for the inhibition of the formation of plaque on teeth.

2. The use of claim 1 in which the pellicular binding site is a carbohydrate receptor.

3. The use of claim 1 in which the pellicular binding site is on a proline-rich protein.

4. The use of claim 1 in which the pellicular binding site is on statherin.

5. The use of claim 1 in which the pellicular binding site is bound more strongly by the binding protein than by the bacterial adhesin.

6. The use of claim 1 in which the binding protein is at least 50% homologous with bovine pancreatic trypsin inhibitor.

7. A process for obtaining a gene encoding a binding protein which competes with a bacterial adhesin for a pellicular binding site and thereby inhibits the formation of plaque which comprises:

(a) preparing a population of cells or phage each bearing a gene encoding a potential binding protein and each bearing on its outer surface the potential binding protein encoded by said gene, said population collectively providing a plurality of different potential binding proteins;

(b) screening said cell or phage-bound potential binding proteins for binding to an immobilized substance presenting said pellicular binding site; and

(c) recovering the cells or phage which bound through said potential binding proteins to said immobilized substance, said cells or phage bearing a gene encoding a binding protein which binds to a pellicular binding site.

8. The process of claim 7 wherein the binding protein genes of said population of cells are mutants of a gene encoding bovine pancreatic trypsin inhibitor.

9. The process of claim 7 wherein the immobilized substance is a proline-rich protein conjugated to hydroxyapatite.

10. The process of claim 8 wherein the proteins are first screened for absence of binding to albumin-blocked hydroxyapatite to which proline-rich proteins are not conjugated.

11. The process of claim 7 wherein plaque bacteria which bind to the pellicle are first incubated with said immobilized substance to saturate the pellicular binding sites, whereby the binding proteins which are bound to said substance are those having a higher affinity for said sites than do the bacteria.

12. A non-naturally occurring protein which inhibits the formation of plaque by competing with a bacterial adhesin for a pellicular binding site, said protein being obtained by the process of claim 7.

13. Use of a binding protein which binds to the active site of a streptococcal glucosyltransferase and thereby inhibits said glucosyltransferase in the manufacture of a composition for inhibiting the formation of plaque.

14. The use of claim 13 in which said binding protein comprises a plurality of GTF inhibitory peptides concatenated by linker peptides.

15. A process for obtaining a gene encoding a binding protein which binds to the active site of a streptococcal glucosyltransferase which comprises:

(a) preparing a population of cells or phage each bearing a gene encoding a potential binding protein and each bearing on its outer surface the potential binding protein encoded by said gene, said population collectively providing a plurality of different potential binding proteins;

(b) screening said cell or phage-bound potential binding proteins for binding to an immobilized substance presenting said pellicular binding site; and

(c) recovering the cells or phage which bound through said potential binding proteins to said immobilized

substance, said cells or phage bearing a gene encoding a binding protein which binds to a pellicular binding site.

16. An anti-plaque agent comprising a pellicle-binding protein conjugated to an enzyme having a plaque-inhibitory effect.

17. The agent of claim 16 wherein the pellicle-binding protein is one which specifically binds to a proline- rich protein of the pellicle.

18. The agent of claim 16 wherein the enzyme is one which hydrolyzes the polysaccharide matrix of plaque.

19. The agent of claim 16 wherein the enzyme is one which catalyzes a reaction which directly or indirectly results in production of a chemical toxic to plaque-forming bacteria.

20. A prophylactic dental composition which comprises a protein which inhibits the formation of plaque by competing with a bacterial adhesin for a pellicular protein binding site, and an orally acceptable excipient.

21. A prophylactic dental composition which comprises a non-naturally occurring protein which inhibits a plaque forming bacterial enzyme, and an orally acceptable excipient.

22. The composition of claim 20 wherein the protein is a preferentially binding mutant of a protein which does not preferentially bind to a pellicular protein binding site.

23. The composition of claim 21 wherein the protein is a preferentially binding mutant of a protein which does not preferentially bind to the active site of a plaque-forming bacterial enzyme.

24. The use of claims 1 or 13 wherein the binding protein is a mini-protein such as CMTI-I or CMTI-III.

25. The process of claims 7 or 15 wherein the binding protein is a mini-protein such as CMTI-I or CMTI-III.

FIG 1A.

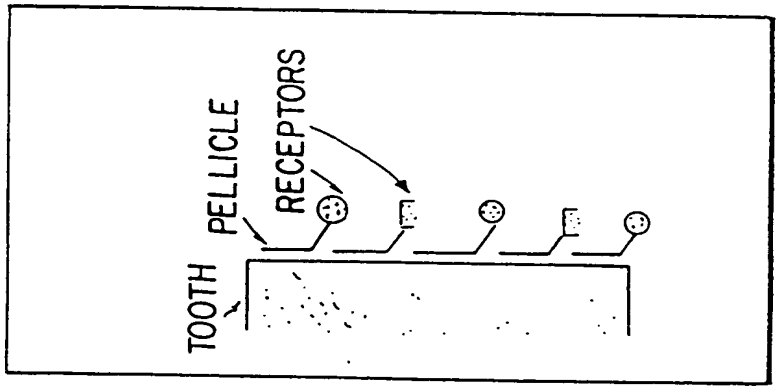


FIG 1B.

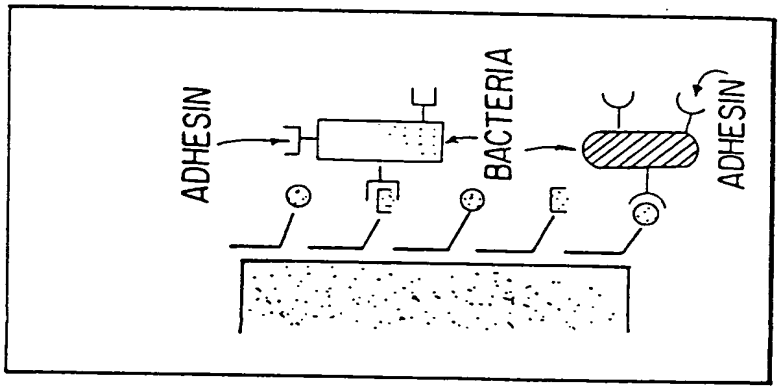
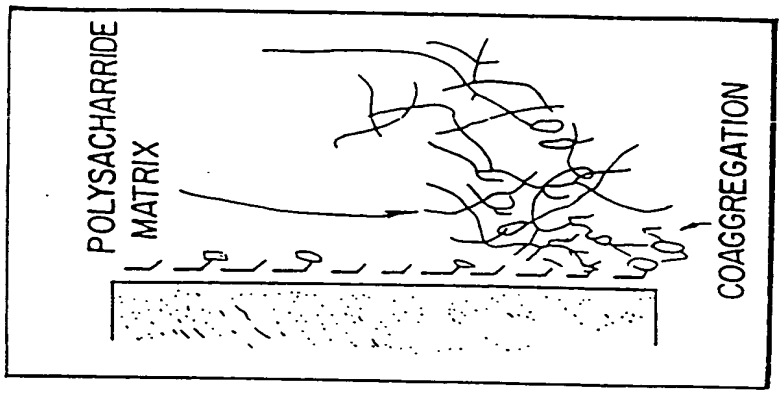


FIG 1C.



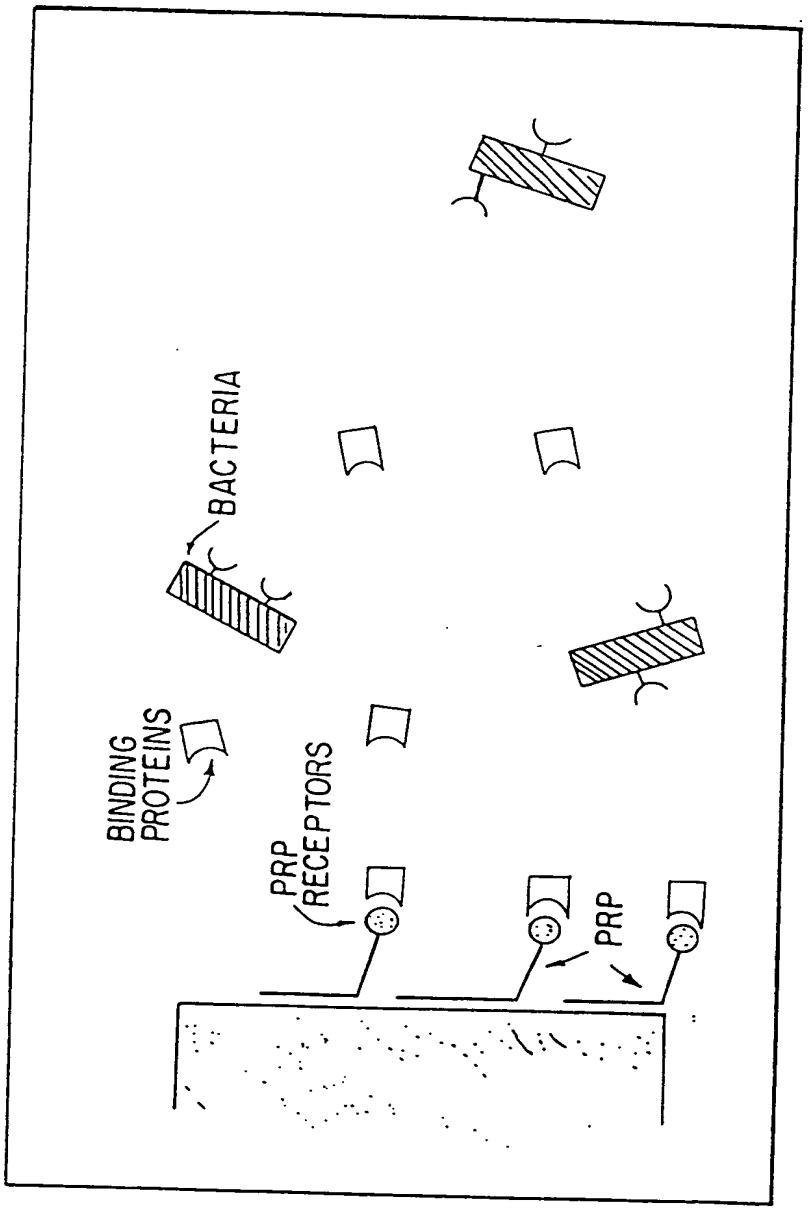


FIG. 2.

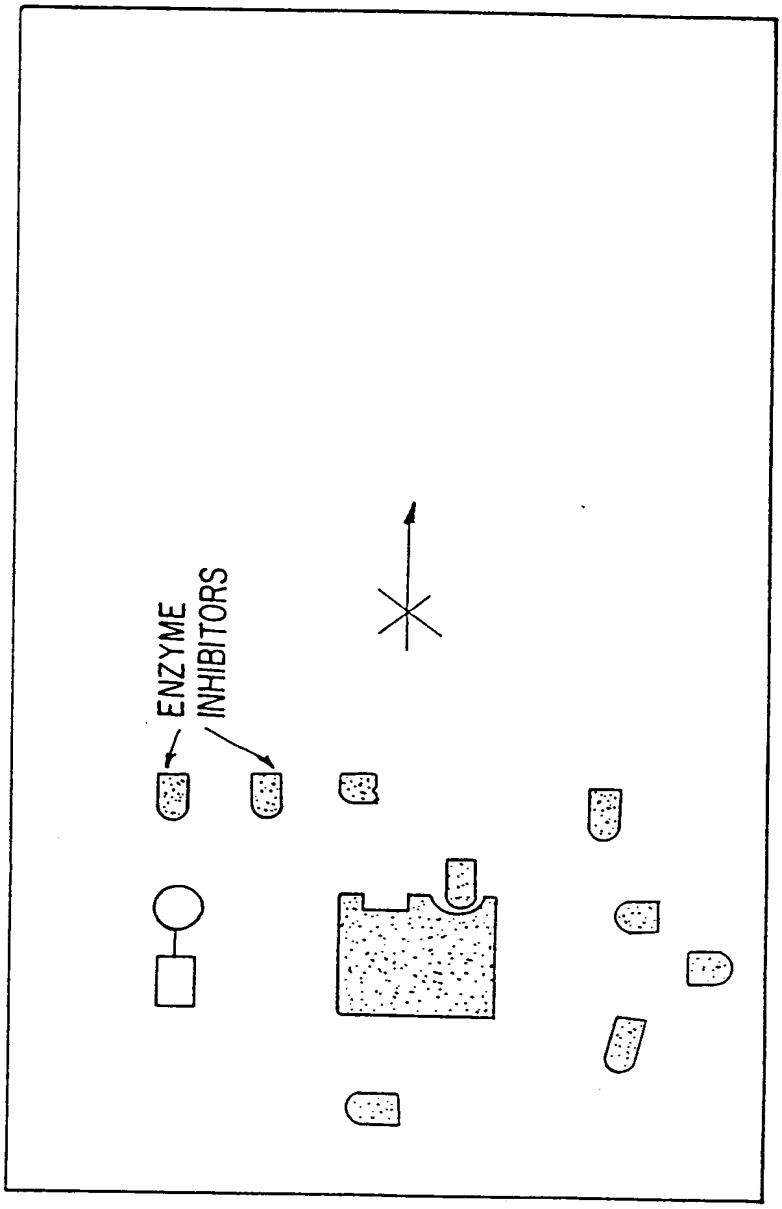


FIG. 3B.

FIG. 4B.

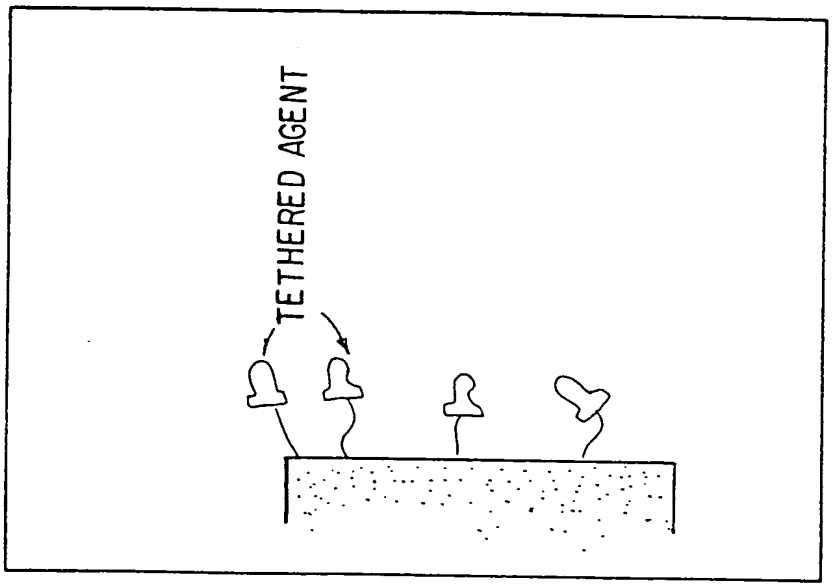


FIG 4A.

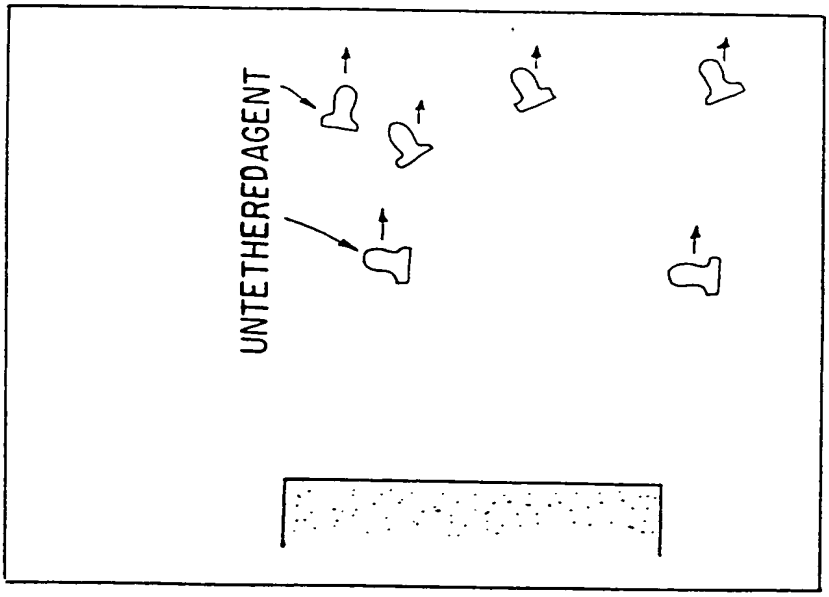


FIG. 5B.

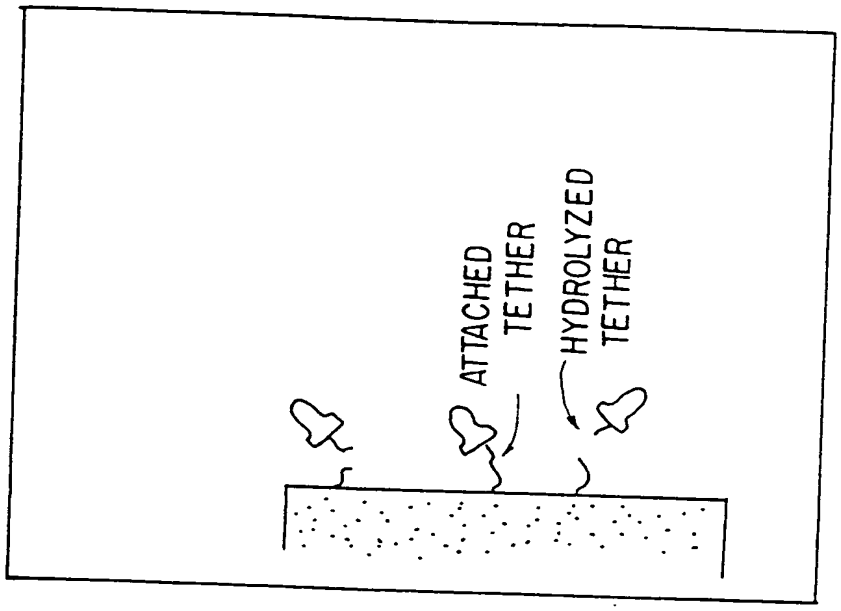

ATTACHED TETHER
HYDROLYZED TETHER

FIG. 5A.

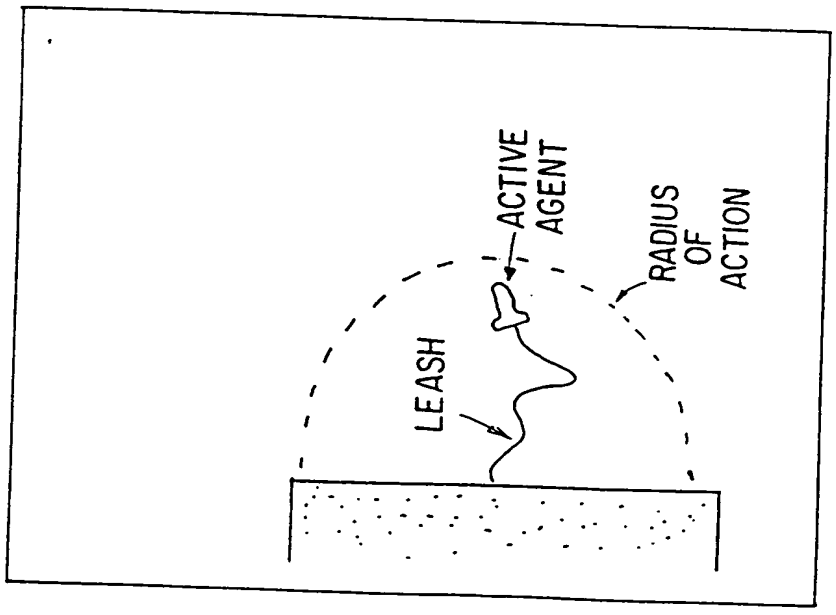

ACTIVE AGENT
RADIUS OF ACTION
LEASH

FIG 6

SUBSTITUTE SHEET

PRIMARY STRUCTURE OF A HUMAN ACIDIC PROLINE-RICH PHOSPHOPROTEIN. THE STRUCTURE SHOWN IS DESIGNATED AS PRP-1. SEVERAL OTHER CLOSELY RELATED PRP DIFFER FROM PRP-1 EITHER BY SUBSTITUTIONS AS RESIDUES 4 AND 50 OR BY POSTTRANSLATIONAL MODIFICATION, AS FOLLOWS.

	RESIDUE 4	RESIDUE 50	MOLECULAR SIZE RESIDUES
PRP-1	ASP	ASN	150
PIF-[SLOW]	ASN	ASP	150
PRP-2	ASP	ASN	150
PRP-3	ASN	ASP	106
PIF-[FAST]	ASP	ASN	106
PRP-4	ASP	ASP	106

PRP-3, PRP-[FAST] AND PRP-4 ARE CONSIDERED TO BE FORMED BY POSTTRANSITIONAL CLEAVAGE OF THE LARGER PROTEINS AT THE BOND ARG 206 GLY 207 PCA, RESIDUE 1, IS PYRROLIDONE CARBOCYLIC ACID, DERIVED FROM GLUTAMATE BY PYRROLIDONE RING FORMATION, A SPONTANEOUS REACTION WHICH OCCURS WHEN GLUTAMATE OCCUPIES THE AMINOTERMINUS.

FIG 6 CONT.

FIG. 7B.

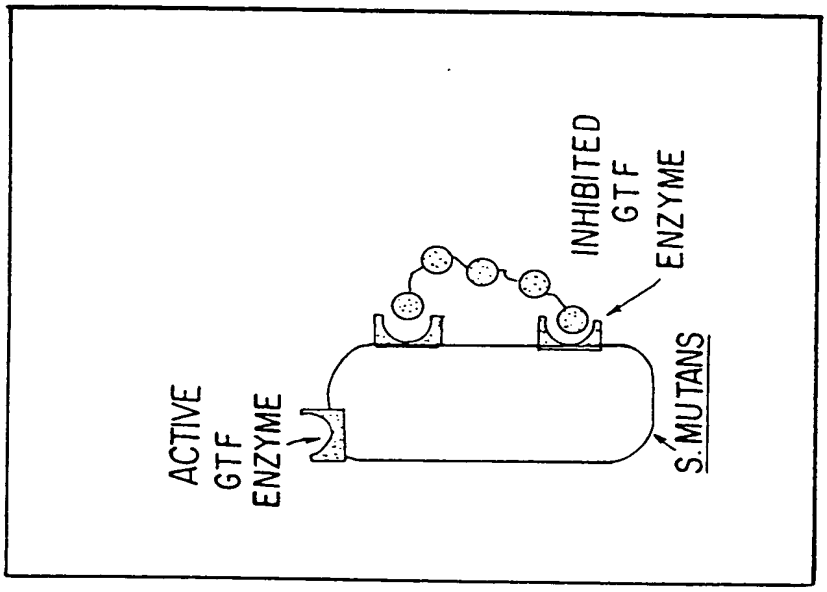

ACTIVE GTF ENZYME
INHIBITED GTF ENZYME
S. MUTANS

FIG. 7A.

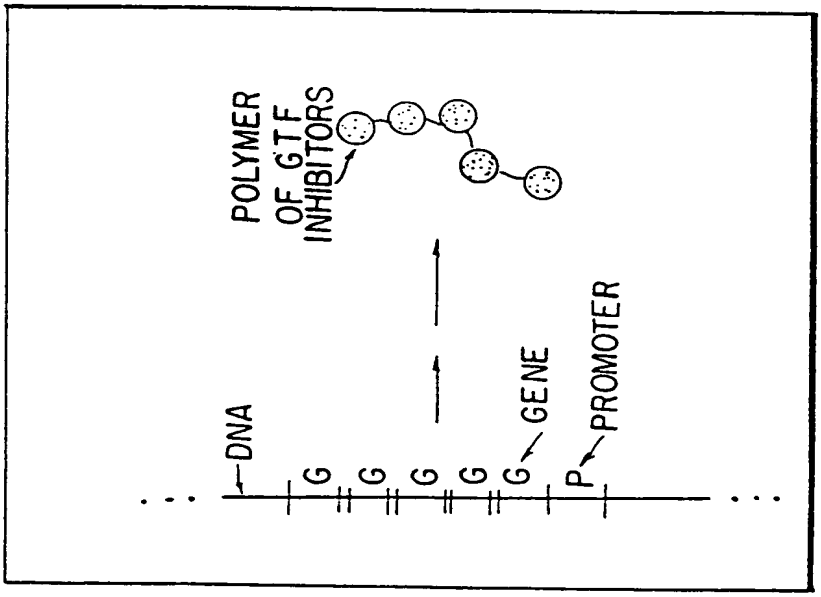

POLYMER OF GTF INHIBITORS
DNA
G
G
G
G
G
GENE
P
PROMOTER

FIG.8.

Suitable gene for production of
Aprotein-derived
PRP-binding proteins

```
5'- GGATCCACTC CCCATCCCCC TGTTGACAAT TAATCATCGG CTCGTATAAT
                          -35    tac promoter      -10

GTGTGGAATT GTGAGCGCTC ACAATTGAGC TCCATggaga aaataaaATG
                                        RBS         M1

AAA CAA AGC ACG ATC GCA CTG GCA CTC TTA CCG TTA CTG
-------- phoA signal sequence ----------------------

TTT ACC CCT GTG ACA AAA GCC CGT CCG GAT TTC TGT CTC
--------------------------> <- aprotinin-derived protein

GAG CCA CCA TAC ACT GGG CCC TGC AAA GCG CGC ATC ATC
-------- aprotinin-derived protein ----------------

CGC TAT TTC TAC AAT GCT AAA GCA GGC CTG TGC CAG ACC

TTT GTA TAC GGT GGT TGC CGT GCT AAG CGT AAC AAC TTT

AAA TCG GCC GAA GAT TGC ATG CGT ACC TGC GGT GGC GCC

TAG TAA TGA TAG GGT TAC CAG TCT AAG CCC GCC TAA TGA

GCG GGC TTTTTTTTTG
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INTERNATIONAL SEARCH REPORT

International Application PCT/US 91/07099

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/10; C07K15/00; A61K7/16; C12P21/00
A61K37/64

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; A61K ; C12P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO,A,9 002 809 (PROTEIN ENGINEERING CORPORATION) 22 March 1990 cited in the application see page 10, line 30 - page 13, line 20	7-8,12
Y	see page 42, line 13 - page 44, line 2 see page 103, line 7 - page 115 see page 122 - page 146, line 5; claims	15,20-22

X	PATENT ABSTRACTS OF JAPAN vol. 8, no. 283 (C-258)(1720) 25 December 1984 & JP,A,59 152 309 (SUNSTAR K.K.) 31 August 1984	20
Y	see abstract	16-20,22

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* Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 JANUARY 1992	17. 01. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 14, no. 68 (C-686)(4011) 8 February 1990 & JP,A,1 290 700 (GODO SHIYUSEI K.K.) 22 November 1989	13
Y	see abstract	15,21

Y	US,A,4 578 265 (PELLICO, M.A. ET AL.) 25 March 1986 cited in the application see the whole document	16-19

A	BIOCHEMISTRY. vol. 28, no. 14, 1989, EASTON, PA US pages 5708 - 5714; MCWHERTER, C.A. ET AL.: 'Novel inhibitors of human leukocyte elastase and cathepsin G. Sequence variants of Squash seed protease inhibitor with altered protease selectivity' cited in the application see the whole document	24-25

A	US,A,4 217 341 (SUDDICK, R.P. ET AL.) 12 August 1980 see the whole document	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9107099 SA 52251

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 08/01/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9002809	22-03-90	AU-A- 4308689	02-04-90
		EP-A- 0436597	17-07-91
US-A-4578265	25-03-86	CA-A- 1167381	15-05-84
		JP-A- 62213754	19-09-87
		US-A- 4537764	27-08-85
US-A-4217341	12-08-80	None	